FACTORS INFLUENCING GROWTH AND METAMORPHOSIS OF THE SALIVARY GLAND IN DROSOPHILA

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Introduction

The present investigations are concerned with the development of the salivary glands in Drosophila. The larval salivary glands are strictly larval organs and grow by increase in cell size. They are completely histolysed during the early part of pupal life. The salivary glands of the adult fly, on the other hand, are imaginal organs which develop from imaginal discs situated at the extreme proximal end of the larval salivary gland, and grow by cell multiplication. Thus both larval and imaginal salivary glands undergo a period of growth during larval life, while metamorphosis leads to the differentiation of the imaginal and the destruction of the larval salivary glands. From the work of Hadorn (1937) we know that the principle causing pupation in Drosophila is a hormone released by the ring gland, a small glandular organ situated dorsally between the two hemispheres of the larval brain. The role of the ring gland in the further differentiation of the various organs involved in the process of metamorphosis is, however, still obscure. Furthermore, virtually nothing is known about the causal factors concerned in the growth of organs during larval life. The present study attempts to analyze some of the causes which underlie the visible expression of growth and differentiation in the development of the salivary glands.

Material and Methods

Drosophila virilis was used for this investigation. Experimental animals were kept at a constant temperature of $25° \pm 0.5°$ C. Instead of the usual method of transplanting larval tissues into larvae, the body of the adult fly was used as a carrier of the larval transplant. The larval tissues transplanted into the body cavity of the adult fly live in their new environment for a long time, perhaps indefinitely. They do not lose their developmental potencies; they grow and differentiate normally when provided with the appropriate stimulus. The adult hosts withstand the operation, which is simple in method, very well. The mortality rate is negligible even when the same host is used for continued transplantations. Indeed this new method approximates tissue culture more closely than any other so far known for insects. Its great advantage lies in the fact that one is able to study the developmental behavior of larval tissues outside their own larval environment. All histological observations on the salivary gland are based on orcein-stained material which was examined either in total mounts or in smear preparations. For certain developmental characteristics the glands were also examined in the living condition.

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Normal Development of the Salivary Gland

In the normal development of the salivary gland one is confronted with a sequence of developmental steps, each of which is characteristic for a definite stage of development. Since the normal development of the salivary gland is necessary for the understanding of the experimental part of this paper, it will be discussed briefly. However, only such features of development will be set forth as are of importance for our special problem.

The salivary glands are paired organs. They develop as invaginations on either side of the anterior ectoderm. As development proceeds these invaginations elongate, grow inward, and unite medially into a common duct leading to the pharyngeal cavity. (Sonnenblick, 1940.) When the embryo hatches, the number of cells in each gland is, according to Makino (1938), about 115 and it does not change during larval life. The growth of the larval gland is, as already mentioned, the result of increase in cell size rather than cell multiplication. The development of the salivary gland has been staged from the time the larva leaves the egg to the time the gland is histolysed. Eleven successive stages of development are shown in Plate I, which represent photomicrographs of salivary gland total mounts, stained with orcein. In Plate II the distal portion of the same glands is shown at a higher magnification. Camera lucida drawings at a magnification of 85× were made for each gland of this normal series, as well as for the glands used in the experiments, and their circumference measured with a planimeter. The size values thus obtained together with special histological landmarks characteristic for the different developmental stages, assured great accuracy in the determination of the various stages. In the following the measurements of the circumferences are given for the normal series of development.

stage of development	1	2	1st molt	3	4	2nd molt	5	6	7	8	9	10–11
circumference (× 85) in cms.	2.5	4.0	6.0	9.0	11.0	12.5	14.0	17.0	23.0	28.0	41.0	meta.

The histological changes that occur during the development of the salivary gland are now set forth. The observations are based on orcein-stained total mounts.

Stage 1 (Plate I, Figure 1 and Plate II, Figure 1′).

The cell size is uniform throughout the gland. The cells are very small and the cell borders not clear. No details are visible in the nucleus.

PLATE I. Normal developmental series of the larval salivary gland of Drosophila virilis. Figures 1 to 11: Photomicrographs of 11 successive stages of development. (Orcein stained total mounts.) Full arrows indicate time of molting. Broken arrow shows position of imaginal salivary gland anlage. (For explanation see text.)

Stage 2 (Plate I, Figure 2 and Plate II, Figure 2′).

The cell size is uniform throughout the gland. The chromatin forms a fine net-work around the edge of a clear area in the nucleus. The chromocenter is visible in all cells and stains deeply.

First molt. The cells are larger than in stage 2; otherwise there is no change.

Stage 3 (Plate I, Figure 3 and Plate II, Figure 3′).

The cells are larger; otherwise unchanged.

Stage 4 (Plate I, Figure 4 and Plate II, Figure 4′).

The cells are larger; they are still uniform in size throughout the gland. The chromatin forms a net-work consisting of fine strands around the edge of a clear area in the nucleus. The chromocenter is visible in all cells and stains deeply. At the proximal tip of the gland, where it borders the duct, a small number of tiny cells becomes visible; these represent the imaginal anlage of the adult salivary gland. (See Plate I, Figure 4, arrow.)

Second molt. The cells in the distal portion of the gland have become larger in size than those of the proximal portion. The chromosome strands, especially those in the nuclei of the distal gland portion, have become wider; alternating deeply stained areas and lightly stained areas are clearly distinguishable within the individual strands. The deeply staining chromocenter is still visible in all cells. The imaginal anlage of the adult gland has become very clear and its cells form a ring-like structure around the salivary duct.

Stage 5 (Plate I, Figure 5 and Plate II, Figure 5′).

The cells in the proximal gland portion are much smaller than in the distal; this difference is very pronounced. The chromosome strands have become wider and the chromatin bands more distinct. The chromosome strands on the periphery of the nucleus embrace the clear area in the center of the nucleus like octopus arms. The chromocenter is still visible in all cells. The imaginal ring cells have increased in number.

Stage 6 (Plate I, Figure 6).

The cells are larger; otherwise unchanged.

Stage 7 (Plate I, Figure 7 and Plate II, Figure 7′).

The difference in cell size between the proximal and distal gland portions is very clear. The chromosome strands are broad and the chromatin bands very distinct. The chromocenter has become invisible in the cells of about one-half of the distal portion of the gland. To avoid misunderstanding this point has to be made clear. The staining capacity of the chromocenter at this stage has by no means changed considerably, as smear preparations show. It has become invisible in total mounts because the chromosome strands in the distal cells have grown so much in width as to eliminate the former discrepancy in width between chromocenter and strands. Therefore if we speak in the following of the disappearance of the chromosome center, we mean just this fact. The disappearance

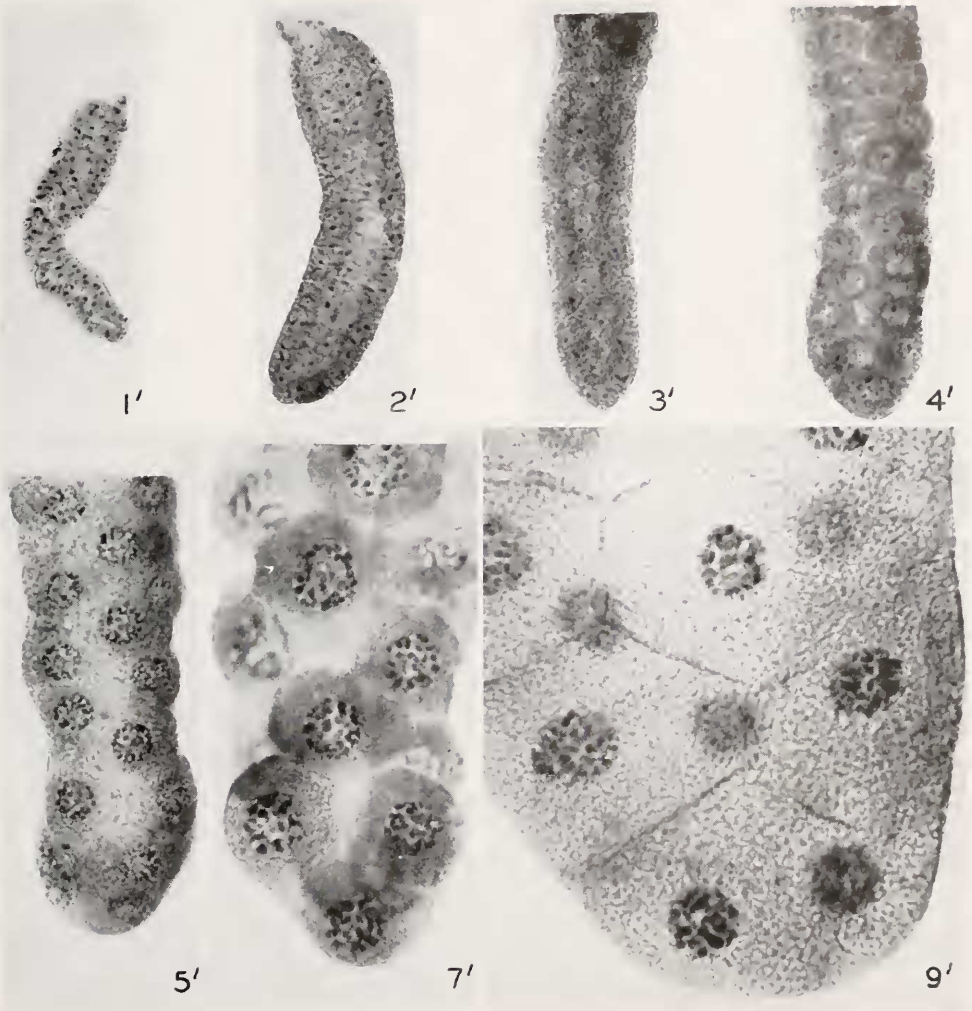

PLATE II. Figures 1′ to 9′: the distal portion of the same glands as shown in Plate I, Figure, 1 at a higher magnification. (For explanation see text.)

of the chromocenter is hence a good indication of the nuclear size. The imaginal ring cells have increased in number.

Stage 8 (Plate I, Figure 8).

The difference between the cell size in the proximal and distal part of the gland is very pronounced. The proximal cells have reached a size which corresponds approximately to the cell size in the distal part of the preceding stage. At no time, however, do we find any sharp separation into a proximal and distal portion of the gland as far as cell size is concerned. On the contrary, the cells increase gradually in size in a proximal-distal direction. Because of this differential growth, the salivary gland acquires its characteristic shape. The form and size of the salivary gland seem thus to be determined by two different growth rates in two different directions; one correlated with age affects the gland as a whole, and the other constitutes a proximal-distal gradient which determines the size of the cells throughout the length of the gland. While up to this time cell growth took place by an increase in size of the nucleus and cytoplasm simultaneously, at this stage a remarkable change occurs in the cells of the distal half of the gland. The nuclei in these large distal cells cease to grow, or grow only very little indeed, yet the cytoplasm increases immensely. A comparison of (Plate II) Figure 7′ with 9′ illustrates this point quite clearly. The chromocenter has become invisible in the distal two-thirds of the gland and the chromatin bands in the chromosome strands especially of the distal cells stain very distinctly. The imaginal ring has increased in size.

Stage 9 (Plate I, Figure 9 and Plate II, Figure 9′).

The larvae are full grown, they have left the food and are ready to pupate. In the living condition the cytoplasm of the glands has become slightly opaque instead of being transparent as in previous stages; this condition is the first indication of metamorphosis. The opaqueness is usually somewhat more pronounced distally. The proximal cells are still smaller than the distal ones. The cells in the distal two-thirds of the gland are extremely large, due to an increase in their cytoplasm, while their nuclei are of about the same size as in stage 8. Although the nuclei are unaltered in size, their condition has changed. The clear area in the center of the nucleus has disappeared. The chromosome strands have become more compact, winding their way through the whole nucleus. The individual strands show their characteristic banding most perfectly. The cytoplasm stains less heavily than in previous stages, while the nuclei take the stain exceedingly well. One of the characteristic features of this stage is that in total mounts the nuclei stand out clearly against a lightly stained background (Figures 9 and 9′). The chromocenters are visible only in the proximal one-fifth portion of the gland. The imaginal ring has become larger; the chromosome strands stretch very well in smear preparations, in contrast to earlier or later stages. This stage is hence best suited for studies on the arrangement of bands in the chromosome strands.

Stage 10 (Plate I, Figure 10).

Histolysis of the glands begins about 10 hours after puparium formation. In various regions, usually first in the distal part of the gland, the cells begin to

vacuolate and the cell walls rupture, while the nuclei are still intact. The cytoplasm in these regions of degeneration stain poorly. The chromosome strands on the other hand stain very deeply. They are clumped in the center of the nucleus and are surrounded by a clear spherical area (Figure 10). In living conditions the glands appear much more opaque, while milky-white zones indicate the regions of advanced histolysis. The chromocenter is by now visible only in a few cells at the extreme proximal end of the gland. The imaginal ring has increased in size.

Stage 10–11 (Plate I, Figure 11).

The next stages of histolysis proceed very rapidly. The degenerating regions within the gland which stain poorly, and which in life appear milky, extend and become more frequent. The nuclei become picnotic. The basement membrane which surrounds the gland breaks down. Finally, about 25 hours after puparium formation, with the probable help of phagocytosis, the larval gland is dissolved. The proximal part is the last to disappear. The differentiation of the imaginal ring begins and is completed during the rest of the pupal period, leading to the formation of the adult salivary gland.

Experiments

The induction of premature metamorphosis.

The description of normal development has revealed that the metamorphosis of the salivary gland consists of the histolysis of the larval and the differentiation of the imaginal salivary gland. The question now arises whether the characteristic developmental behavior of the gland during metamorphosis is dependent on or independent of the conditions prevailing in the animal during metamorphosis. This can be tested by transplanting young salivary glands into older hosts, thus exposing the gland prematurely to the metamorphosis factors.

Salivary glands of stage 5 were transplanted into the abdomen of older larvae shortly before pupation. The transplanted glands were dissected and their condition studied after they had remained in the hosts for various lengths of time. The results of the experiments are summarized in Table I A. The first animals in this series were dissected two days after the operation, when the hosts were about one-day old pupae. The transplanted glands had reached stage 10, i.e. they showed clear signs of histolysis. Their state of development corresponded closely to that of the normal host gland. Yet if the transplanted glands had been left in their own environment, they would have developed certainly not further than stage 8 by this time. Thus, under the influence of the metamorphosis factors of the host, the grafted glands have metamorphosed prematurely. The transplanted glands reached an advanced stage of histolysis when they were left three days in the host, and they disappeared completely when left in the host for six days or longer.

In these transplantations the anlage of the imaginal ring was included in the larval graft. Now it was found (Table I A), that the imaginal gland, like the larval gland, is able to metamorphose precociously when exposed to the metamorphosis factors prematurely. This becomes evident from the observation that the transplanted imaginal anlage in the newly emerged host, i.e. six to seven

days after the operation, is already completely differentiated into the imaginal salivary gland (Plate III, Figure 4), while donor controls by this time would have been two days old pupae with quite undifferentiated imaginal glands.

In summarizing the experiments at this point, we find that the metamorphosis of purely larval structures, as well as imaginal structures, is not autonomous,

TABLE I A

Transplantation of young salivary glands into larvae shortly before pupation.

A. Transplantations of larval glands of stage 5.

Circumference of transplanted gland	Days transplant remains in host	Host stage dissected:	Stage of transplanted larval gland	Stage of transplanted adult gland
14.0	2	pupa	stage 10+	larval
15.0	2	pupa	stage 10−	larval
15.0	2	pupa	stage 10+	larval
15.5	2	pupa	stage 10−	larval
15.5	2	pupa	stage 10−	larval
13.5	3	pupa	stage 11−	larval
13.5	3	pupa	stage 11−	larval
15.5	3	pupa	stage 11+	larval
15.5	3	pupa	stage 11−	larval
15.5	3	pupa	stage 11−	larval
13.5	6	adult	completely histolysed	adult gland formed
13.5	7	adult	completely histolysed	adult gland formed
14.0	7	adult	completely histolysed	adult gland formed
13.5	8	adult	completely histolysed	adult gland formed
13.5	10	adult	completely histolysed	adult gland formed
15.5	13	adult	completely histolysed	adult gland formed

TABLE I B

B. Transplantations of larval glands of stage 3.

7.5	2	pupa	stage 6	larval
7.5	3	pupa	stage 10−	larval
7.5	3	pupa	stage 10−	larval
9.0	3	pupa	stage 11	larval
7.5	6	adult	stage 11−	larval
7.5	6	adult	stage 11−	larval
7.5	6	adult	stage 11	larval
7.5	7	adult	stage 11−	larval
9.0	9	adult	stage 11	larval
9.0	9	adult	stage 11	larval
9.0	9	adult	stage 11	larval
9.0	9	adult	stage 11	larval
7.5	10	adult	stage 11	larval
7.5	15	adult	stage 11	larval

but is caused by some factors in the organic environment of the host during the period of metamorphosis.

The next question to be answered is whether salivary glands younger than stage 5 are competent to respond to the metamorphosis factors. For this,

salivary glands of stage 3 were transplanted into older larvae shortly before pupation. The region of the imaginal discs was again included in the graft, but in contrast to the previous series of experiments the imaginal ring as such was not yet morphologically visible. The results of these experiments summarized in Table I B show that histolysis of the young transplanted glands begins about two days after the operation, but is never completed, although the glands may remain as long as 15 days in the host. Young glands which have remained for three days in the host only begin their histolysis, while older glands are at this time quite extensively histolysed (Cff. Table I A with I B). Thus the young glands are presumably unable to respond at a time when the metamorphosis factors are most effective. We must assume that the metamorphosis factors have become less efficient when the glands have finally reached their responsive stage, and are hence unable to induce complete metamorphosis, because it is difficult to understand why the glands should not be histolysed completely if the metamorphosis factors were still active in the late pupal or adult stage.

The inability of the young organ to respond to the metamorphosis factor is demonstrated in the behavior of the adult gland (Table I B). In spite of the fact that the young imaginal gland discs have remained in some cases for a considerable length of time in the host, they show no signs of metamorphosis (Plate III, Figure 3). Although the anlagen have developed well beyond their stage of transplantation and have acquired their typical ring-shaped form, they remain larval and never surpass this stage. Their state of development corresponds to stages 10 and 11 of the normal developmental series, yet this stage, as previous experiments have shown, by far exceeds the stage at which the adult gland anlage is able to react. These observations lead necessarily to the conclusion that the larval development of the disc is not interrupted in its new environment, but that by the time the disc has reached its reactive stage the factors necessary for promoting metamorphosis are absent or not effective enough. The specific results of this experimental series may then be briefly summarized as follows: the larval glands, as well as the analgen of the adult glands, must reach a certain developmental stage before they are able to respond to the metamorphosis factors. If they reach this stage after the active period of metamorphosis they are unable to metamorphose and persist as larval structures in an imaginal environment (Plate III, Figure 3).

In examining prematurely-metamorphosed young salivary glands, one has the impression that the nuclei in these glands are histolysed before they have attained their fully normal size. The same observation was made in prematurely-metamorphosed young salivary glands which were grown in the body of adult hosts. It is difficult to decide whether this impression is real, since the reduction of the nuclear size is only slight and probably within the limit of normal variation. If true, however, it would mean that premature metamorphosis causes an early cessation of nuclear growth, which is not at all unlikely. In the light of these considerations, it seemed interesting to investigate whether older glands transplanted into younger hosts would grow larger than normally, because under these experimental conditions they would be exposed to the metamorphosis factor later than normally. Salivary glands of stage 7 were therefore transplanted into younger host larvae, the salivary glands of which were at stage 4 at the time of the operation. In other words, salivary glands of the last larval instar were

transplanted into hosts of the second larval instar. This experimental series consisted of six hosts which were dissected two days after the operation when the transplanted and host salivary glands were compared. It was found that the host salivary glands had developed to stage 8−. They were still transparent, and showed no signs of metamorphosis. The transplanted glands on the other hand were found to be at stage 9+ and had begun to metamorphose, as indicated by their intensely opaque appearance. Moreover, the chromosome strands of the transplanted gland were distinctly wider than those of the host glands. Yet the strands were definitely not larger than normally, i.e. their size was characteristic for a normal salivary gland of stage 9+. Thus the original question whether it is possible to obtain salivary glands larger than normal has been answered by this experiment in a negative way. Some further conclusions which might be drawn here will be discussed on page 30 in a somewhat different connection.

The effect of the ring gland on the development of the salivary gland.

Until now we have dealt almost entirely with specific reactions of the salivary gland. So far nothing has been said about the factors which govern the processes of growth of the gland during larval life and those of metamorphosis during pupation. In the light of our knowledge of hormone-controlled insect metamorphosis, we expect these factors to be hormonal in nature. The search for the activating principle resolves itself into the problem of locating an organ or organs of internal secretion and demonstrating their action on the developing organ system. There are obviously two alternatives in attacking this problem experimentally. One may attempt to remove systematically various organs of supposedly endocrine nature from the larvae and thus hope to locate the responsible organ by testing the developmental behavior of the operated animal. The difficulty involved in a study of this kind is mainly a technical one, for it is very difficult indeed and at the present time seemingly impossible to perform such an operation in the larvae of Drosophila. The other possible approach open to the investigator is to dissect various organs from a donor larva which can be sacrificed and transplant them to a second larva and then test their effect on the development of the host. Although technically quite feasible, this procedure has one great disadvantage. The effect of the transplanted gland on the host may be counteracted or blurred to a great extent by the hormone supply of the host itself, and thus not detectable. The introduced hormone, moreover, may upset the host system to such an extent that the situation, instead of being clarified, is rather obscured. Apart from these difficulties there is the additional one that both approaches are rather indirect, since the experiments are designed

PLATE III, FIGURE 3. Adult salivary gland, developed from the imaginal salivary gland anlage which was transplanted at stage 6 into a host larva shortly before pupation. Transplant dissected from the emerged host six days after the operation.

FIGURE 4. Larval salivary gland persisting as larval structure in the adult host; obtained by transplanting salivary glands at stage 3 into the abdomen of host larvae shortly before pupation. Note: undifferentiated imaginal ring cells (arrows).

FIGURE 5. Paired salivary gland transplantation. Glands transplanted at stage 6 into adult hosts and dissected two days after the operation. *a*. Partner transplanted alone: note bloated appearance of gland and the small nuclear size. *b*. Other partner transplanted together with two ring glands. Note the large, very well stained nuclei with chromosome strands.

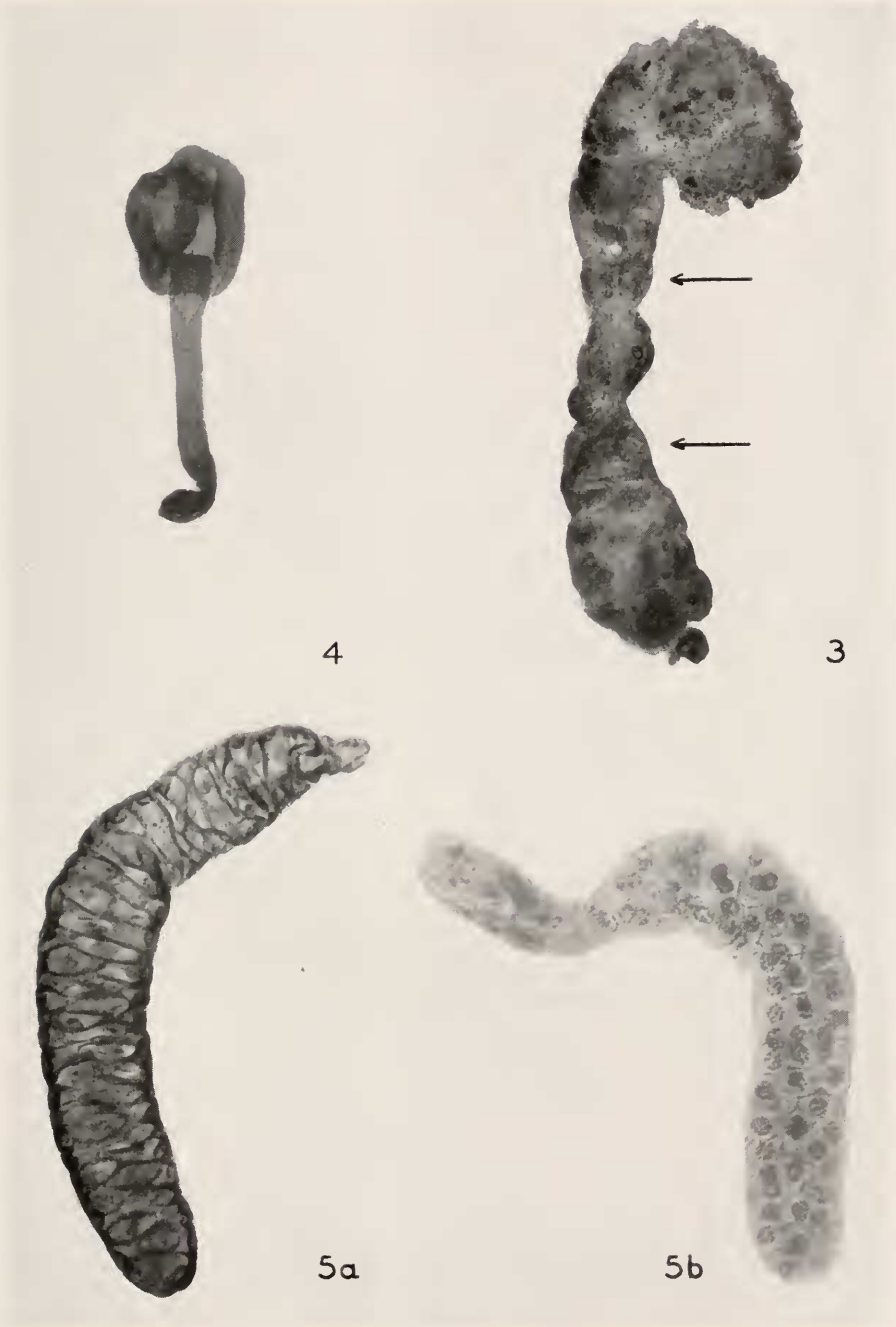

PLATE III

for testing the hormone action on the whole organism rather than on the special organ. We have hence to seek a method by which the hormone action may be more directly studied.

An effort in this direction was made by using the abdominal cavity of the adult male fly as a place for culturing larval tissue in vitro. This environment is supposedly neutral as far as the progress of development is concerned, for the larval salivary glands cease to grow after the transplantation, but continue to live indefinitely. The transplanted tissues do not suffer from a lack of nutrition nor are they unable to utilize the nutritive components of the new environment, as is shown by the following experiment. When young Drosophila larvae are starved, they use the fat stored in their fat bodies and, if starvation is continued, they exhaust their food reserves in the fat bodies completely. Such exhausted fat bodies are strikingly different from the fat bodies of feeding larvae. Now it was found that starved exhausted larval fat bodies restore their food reserves again and become indistinguishable from fat bodies of normally fed larvae when transplanted into the body cavity of adult flies. For these experiments a small strip of fat body closely attached to the salivary gland was transplanted together with the gland. The gland, important in this instance only as a marker, enables one to find and distinguish the transplanted fat body from the fat bodies of the host. The inability of the larval organs to develop in the adult fly is hence not caused by a lack of nutrition but obviously by the lack of some other factor.

Before considering the nature of this developmental factor, one further point has to be cleared up. It has to be shown whether the inability of the gland to develop is typical for glands of all ages or only characteristic for glands of a particular stage of development. In order to elucidate this point, salivary glands of stages 2 to 6 were transplanted into the abdomen of adult male flies. The grafted organs were allowed to remain for various lengths of time in the host before they were dissected out and their developmental condition examined. The results of these experiments summarized in Table II show clearly the inability of the gland to develop in the body of the adult fly regardless of their age at the time of the transplantation and of the length of time they remained in the host. The only perceptible way in which the transplanted glands seemed to be changed while in the adult host was that they became greatly inflated, this being caused by the accumulation of a clear watery fluid in the lumen of the gland. The longer the glands remained in the host, the more fluid accumulated and the more bloated the glands became. The accumulated fluid presumably represents saliva, which is secreted by the gland cells into the lumen of the gland and is unable to escape, since the outlet is closed off in dissecting the gland from the donor larvae. Incidentally, should this fluid really prove to be saliva, we have here a method of accumulating it in order to study its chemical properties.

The foregoing experiments have shown that the larval salivary glands depend for their development upon some factor missing in the body cavity of the adult fly. This factor was found to be the larval ring gland, which when transplanted simultaneously with the salivary glands into the abdominal cavity of the adult fly, caused the latter to continue their development leading to histolysis. The progress of development of the salivary glands was indicated by changes in the nuclei, cell growth and certain characteristic staining reactions. All the typical developmental stages found and described in the normal developmental series

may be observed, but they proceed at a much lower rate. The salivary glands are also able to metamorphose, i.e. they become gradually histolysed if left long enough in the adult host. The ring glands used for these experiments came from donor larvae which were ready to pupate. The number of ring glands implanted into one host varied from one to four. Salivary glands of stage 2 to stage 6 were tested for their reaction to the ring gland factor. The transplanted salivary glands remained in the host for various lengths of time. These experiments, summarized in Table II, led to the discovery of a number of essential facts concerning the development of the salivary glands. Glands of all ages tested respond to the ring gland factor. The older the gland is at the time of transplantation, the earlier metamorphosis occurs. This implies that at least younger glands do not metamorphose right away, but grow to a certain point before metamorphosis begins. In the study of normal development we have seen that the first signs of metamorphosis are noticeable in glands of stage 9, as indicated by a slight but distinct opaqueness of the gland in living condition. At this time the chromocenter has become invisible in the distal portion of the gland. We find in glands which develop in adult hosts some variability in the relationship between the onset of opaqueness, i.e. metamorphosis, and the disappearance of the chromocenter. The degree of opaqueness observed in the transplanted glands is indicated in Table II by the Roman numerals I to III placed next to the number which designates the stage of development. The number I represents an opaque condition of stage 9 and the number III of stage 11— in normal development. When no Roman numerals are given, the relation between metamorphosis and developmental stage is normal. The transplanted glands become opaque at an earlier developmental stage. This is especially true in the transplantation of younger glands. For instance, glands which have developed in the adult host to stage 7— show an opaque condition which corresponds to stage 9 or 10 in normal development. Since we know from normal development that the chromocenter becomes invisible because of the thickening of the chromatin threads, it follows that the glands begin their metamorphosis before the strands have developed to their normal size. It was also noticed in many cases that the nuclear size in advanced prematurely metamorphosed glands seemed smaller than normal. This would indicate that they degenerated before they had reached their full size. These facts are in agreement with the experiments described on page 19 where young glands in old larval hosts were made to metamorphose precociously. The number of ring glands transplanted seems to be of no great importance for the development of the salivary gland, since one or three ring glands produce about the same effect.

In all these experiments where adult flies were used as hosts the imaginal salivary anlage usually transplanted together with the larval gland remained larval. Its cell number, however, increased considerably when transplanted together with ring glands. The amount of increase depended upon the time the anlage remained in the host. However, no sign of differentiation could be noticed, in spite of the fact that in some cases more than four ring glands were present and the disc remained for a considerable length of time in the host. In the effort to obtain a more precise comparison between the developmental behavior of larval salivary glands with and without ring glands, the two glands of one donor were compared with each other. For this experiment the paired

gland of a single donor was dissected, the two glands separated and one partner transplanted into one adult host without ring glands and the other partner transplanted into a second adult host together with two to four ring glands.

TABLE II

Transplantation of salivary glands of various ages; alone and together with different numbers of ring glands into the abdomen of adult male flies. Roman numerals indicate state of opaqueness of the gland. (For explanation see text.)

Stage of transplanted salivary gland	Days transplant remains in host	Number of ring glands transplanted									
		None		One		Two		Three		Four	
		Number of cases	Stage of development	Number of cases	Stage of development	Number of cases	Stage of development	Number of cases	Stage of development	Number of cases	Stage of development
	2	1	3−	1	I 9						
2	3			2	5						
↓	4	2	3	2	I 7−			1	5		
2	5									1	4+
	7	2	3−					2	6	1	I 7−
	14	1	3−					1	II 9+		
	6	1	3			1	I 7+				
3	9	2	3			2	II 8				
	13	1	3			1	III 10				
	1	1	4					1	5+		
4	4	4	3+	1	I 7			1	I 8−		
↓	6	4	4−	2	I 7					3	10
4	7	4	4−					2	II 8+		
	8	3	4+								
	9	2	4+							1	II 8
	2	1	5−					3	6		
	2	1	5					2	II 9		
	5	1	5							1	I 7
5	6	1	5			1	8				
	7							1	II 9−		
	9	2	5			2	10+				
	10	1	5					1	10		
	13	1	5−			1	11−	1	11−		
	2	5	6			5	9+				
6	4	1	6			1	10−	1	III 9+		
	7	1	6			1	10+	2	10		
	14							1	11−		
Number of cases		45		8		15		20		7	

The gland in the first host thus served as a control for the partner gland in the second host. At the desired time, both hosts were killed simultaneously, the two glands dissected and compared. Twenty-three such pairs are available;

they are recorded in Table III. As in the previous experiments we find the salivary glands unable to develop when transplanted alone, while the partner glands with added ring gland implants continue development. Figure 5 of plate III illustrates such a paired transplantation. This pair was transplanted at stage 6 and left for two days in the adult host. The partner (*a*) transplanted alone, has become greatly inflated, yet has not developed beyond the stage of transplantation. The other partner (*b*) which was transplanted together with

TABLE III

Paired transplantation of salivary glands of different age into the abdomen of adult male flies. Note: one host carries salivary gland alone, while second host carries partner salivary gland and ring gland grafts.

Stage of transplanted salivary gland	Number of ring glands transplanted	Days graft remains in host	Condition of transplanted salivary gland	
			Partner without ring gland stage:	Partner with ring gland stage:
2	4	5	2	4+
3	2	6	3	I 7+
3	2	9	3	II 8
3	2	9	3	II 9
3	2	13	3	10
4	3	1	4	5+
4	3	4	4	I 8−
4	3	7	4+	II 8+
4	3	7	4	III 9+
5	2	6	5	II 9+
5	2	9	5	10−
5	2	9	5+	10+
5	2	13	5	11−
6	2	2	6	9+
6	2	2	6	9+
6	2	2	6+	9+
6	2	2	6	9+
6	2	2	6	9+
6	2	2	6	9+
6	2	3	6	9+
6	2	4	6	10−
6	2	6	6	10+
6	2	7	6	10+

two ring glands has continued its development and has reached stage 9+. Plate III, Figure 5b illustrates clearly the characteristic large nuclei at this stage which stand out against a lightly stained background (Cf. Plate III, Figure 5b with stage 9 of the normal developmental series). The chromocenter has become invisible in at least two-thirds of the gland. The chromosome strands are broad and the chromatin bands within them very distinct. The single cells are extremely large, due to an increase in the volume of the cytoplasm. In the living condition the cytoplasm is opaque in contrast to the transparent

appearance of the partner gland. Moreover, the salivary gland which has undergone development is not swollen as is the case with the partner gland. This fact can be seen in all salivary glands which are transplanted together with ring glands. The ring gland must hence interfere with the secretory function of the salivary gland. On turning again to Table III we find that the first response to the ring gland is obviously a growth response. This becomes evident if one compares glands of the same age which were left for increasingly longer periods in the host. (See Tables II and III.) For example, glands transplanted at stage 4 grow in one day to stage 5+, in four days to stage 8− and reach stage 9+ when left for seven days in the host. After growth has continued for a certain length of time, the physiological condition of the glands must have changed in some way, since they now respond with metamorphosis to the ring gland factor. The younger the glands are at the time of transplantation, the later they respond with metamorphosis, for glands transplanted at stage 2 develop in five days only to stage 4+, while glands transplanted at stage 4 show indications of metamorphosis after four days. Metamorphosis is far advanced in four days when glands of stage 6 are used for the transplantation. The transplanted glands, especially the younger ones, metamorphose precociously, for the opaque metamorphosis condition is noticeable in transplanted glands as early as stage 7+, thus at a considerable earlier stage than in normal development. (See also Table II.) This abnormal relationship between the stage of development and the onset of metamorphosis becomes less pronounced as increasingly older glands are used.

The different responsiveness of younger and older salivary glands has been demonstrated very clearly in a further experiment especially designed for testing this point. Two salivary glands, one of stage 3 and the other of stage 5, were transplanted together with two ring glands into the abdomen of a single adult host. This series consisted of four hosts, each of which contained a young and an old salivary gland, as well as two ring glands. Six days after the operation, the first host was examined. The young salivary gland had developed to stage 7+ and was slightly opaque (I), while the older gland was at stage 9+ and distinctly more opaque. In two hosts examined nine days after the operation, the younger glands were found to be at stage 8 to 9 and the older glands at stage 10+. While the younger glands appeared intensely opaque, the older glands were obviously in an advanced state of metamorphosis, as indicated by large milky regions of degeneration. The last host examined 13 days after the operation revealed the young gland at stage 10 and the older one at stage 10+. Again histolysis had progressed further in the older gland.

In a second series of this kind, consisting of six animals, salivary glands of stages 2 and 3 were transplanted together with three ring glands into a single host. The results of these experiments are essentially the same as in the foregoing series; they show again that older glands are always further metamorphosed than young ones, although, as pointed out before, the onset of metamorphosis in the young glands is premature, relative to their state of development.

It has been seen that the development of the salivary gland in the adult host with the support of the ring gland is considerably slower than in normal development. However, the question as to how much development is slowed down has not as yet been examined. In order to elucidate this point, one partner

of a pair of glands of stage 5 was transplanted into the abdomen of an adult host together with 3 ring glands, while the other partner was transplanted into the abdomen of an old larva shortly before pupation. In this way it was possible to make a direct comparison of the same gland in an adult and larval environment. From six such experimental pairs, five were examined two days after the operation. At this time the larval hosts pupated. In one pair the gland in the larval host was found to be at stage 10, showing clear regions of degeneration, while the partner gland in the adult host had only developed to stage 9 and appeared but slightly opaque. In two other pairs only the proximal part of the gland in the pupated larval host could be found, since the distal gland region was completely histolysed. The partner glands in the adult host in one of these pairs had developed to stage 9, being slightly opaque, while the gland in the other adult host had reached only stage 8 and was still transparent. The glands in the pupated larval hosts of the two remaining pairs could not be found, presumably because they were already completely histolysed. The partner glands in the adult hosts were found to be transparent and had reached stage 8. The last pair of this series was examined seven days after the operation. At this time the larval host had already emerged. Its graft could not be found and was apparently completely histolysed. The partner graft in the adult host was at stage 10−, showing advanced signs of histolysis.

The results of these experiments bring into focus one interesting point. We have seen from the previous experiments that growth and metamorphosis of the salivary gland is controlled by the ring gland. The salivary glands in the larval hosts are under the influence of only one ring gland, namely that of the host, while in the adult hosts the glands are exposed to the effects of two ring glands. In spite of this, we find the progress of development of the salivary glands much more rapid in the larval than in the adult host. This shows that the ring gland factor is more effective in a larval than in an adult environment.

Before closing the experimental part of this paper it should be mentioned that the ring gland factor is not species-specific, since ring glands of melanogaster cause the development of virilis salivary glands in both virilis and melanogaster hosts. Conversely, melanogaster salivary glands develop under the influence of virilis ring glands in virilis or melanogaster hosts.

Discussion

The present data prove without doubt the necessity of the ring gland for the metamorphosis of the salivary gland. The responsible ring gland factor is presumably hormonal in nature, since the transplanted ring glands have no contact with the test organs which are nevertheless compelled to metamorphose. The ring gland is thus the source of the metamorphosis hormone in Drosophila and is not responsible only for puparium formation as previously assumed. In fact, puparium formation has to be considered as the first step in metamorphosis. The observations of Hadorn and Neel (1938) that ring glands implanted into young larvae bring about premature puparium formation but have no effect on the metamorphosis of the larval organs can be explained by the incompetence of the young organs to respond to the metamorphosis hormones. This is supported by the observation that young salivary glands react more slowly to the ring gland hormone than older ones and that the salivary glands must reach a

certain developmental stage before they are able to respond to the metamorphosis hormone. The inability of organs to respond with metamorphosis before a certain stage of development is reached is not peculiar to salivary glands but has been observed for eye discs (Bodenstein, 1939a and b), and for ovaries (Vogt, 1941). Inasmuch as the time at which larval glands and imaginal gland anlagen reach their state of responsiveness is concerned, we find that both structures become responsive at about the same time. This, however, is true only of grafts into larval hosts; in adult hosts we find the larval glands able to metamorphose, but not the imaginal glands, in spite of the presence of several ring glands. This indicates clearly that the larval glands respond much more readily than the imaginal glands. In comparing the ring gland action in a larval and an adult environment, we find the hormone more active in the former. Whether one ring gland in the larval host is able to produce more hormone and thus assure a higher hormone concentration than four ring glands in an adult host is questionable, but would explain easily why in the adult host the imaginal anlage is unable and the larval gland is able to respond; and why in the larval host both glands are able to metamorphose synchronously. On such an hypothesis the low hormone level in the adult host is able to induce metamorphosis only in the readily responsive larval gland but not in the imaginal gland anlage, which needs a higher hormone concentration for the same task. In the larval environment on the other hand, where the hormone concentration is presumable higher, it is sufficient to induce metamorphosis in the imaginal gland also. In the light of these considerations it becomes evident that any difference in the competence of the reactive tissue might be detectable only when low hormone concentrations are used. The quantitative action of the metamorphosis hormone has also been demonstrated by Hadorn and Neel (1938), who found that pupation in Drosophila occurs sooner when three instead of one ring gland are implanted into the larval host. The question of hormone concentrations was not directly tested in our experiments because there seemed to be no difference in the effect of two or more ring glands. There is, however, some indication that one ring gland is not as effective as four; this can be seen if one compares (Table II) salivary glands which were transplanted at stage 4 together with one and with four ring glands and which were then left for six days in the adult host. The salivary glands (two cases) transplanted together with one ring gland have developed to stage 7 and the salivary glands transplanted together with four ring glands to stage 10 (three cases). Similar experiments on other organ discs not reported here gave the same results. Thus it seems quite certain that the hormone concentration is a decisive factor in the development of the salivary gland.

One of the most important facts revealed by the present investigations is that the first salivary gland response to the ring gland hormone is a growth response. It is only after the salivary glands have grown to a certain size that the hormone elicits the metamorphosis response in the salivary gland. The younger the salivary glands are at the time of transplantation the longer the growth period persists. In other words, younger glands begin their metamorphosis later than older glands when under the influence of the same number of ring glands, yet the onset of metamorphosis of young glands in adult hosts under ring gland influence is definitely premature when compared with normal develop-

ment. However young the salivary glands are when transplanted, the onset of their metamorphosis is never earlier than stage 7−, as compared with stage 9 in normal development. This shows that the young glands are able to respond with growth only they have reached stage 7−. This observation leads us directly to the question why in normal development the onset of metamorphosis takes place at stage 9 and not at stage 7−. The answer to this may be found in the following considerations. The experiments have shown that the growth of the salivary glands depends on the ring gland hormone. This must obviously also be the case in normal development. Now in normal development the salivary gland is under the influence of the ring gland during the entire larval period. However, the ring gland of younger larval stages is much smaller than the ring gland of mature larvae and thus probably produces much less hormone. The hormone concentration of younger larvae is consequently expected to be much lower than in older larvae. The hormone level in younger larvae, we might argue, is thus not high enough to assure metamorphosis, but is sufficient to promote growth. As development proceeds, the ring gland grows and thus produces more hormone; at the same time however, we find the competence of the salivary gland changing. At a given time, therefore, when hormone level and responsiveness of glands are in a definite relationship with each other, metamorphosis occurs. In normal development this time is reached when the salivary glands have developed to stage 9. We may now ask why, under experimental condition, we are able to metamorphose glands which have reached only stage 7−. The answer to this lies very probably in the fact that we have changed the hormone concentration. In the adult host the hormone level produced by several ring glands is presumably much higher than in younger larvae and is thus able to induce metamorphosis in glands as young as stage 7−. Since metamorphosis proceeds at a very slow rate, we must conclude that the experimentally established hormone level in adult animals, while higher than in younger larvae, is not as high as in full-grown larvae, where young glands metamorphose much more rapidly. Whether the ring glands produce less hormone in the adult fly, or whether some other factors are responsible for the fact that we are unable to raise the adult hormone level to the equivalent of the level existing in the old larvae is, however, still questionable.

There is one further point of considerable interest. We have seen on page 22 that old salivary glands transplanted into younger larvae metamorphose before the glands of the host larvae show any signs of metamorphosis. Since the cell size of the grafted glands was found to be not larger than normal, there seems to be a limit beyond which nuclear growth is impossible. The question now arises, what exactly do we mean by this limit? Judging from the results cited earlier, we have to assume that the salivary glands become more and more responsive to metamorphosis as they increase in age. The older gland in the young host is therefore at a much more advanced stage of responsiveness than the host glands, and is hence able to react with metamorphosis to a hormone level much lower than that needed for the same reaction by the younger gland. In assuming, as we have, that the hormone level increases gradually during the larval period, the growth limit is nothing more than the expression of a definite relationship between cell competence and hormone level. In the light of this it is theoretically possible to obtain cells larger than normal, when older glands

are affected by a hormone level too low for metamorphosis but high enough for growth; granted, however, that a hormone level low enough for a highly responsive gland could be found.

Logically this conception is based on the assumption that the ring gland produces its hormone during the entire larval life. Only with the demonstration of this does the present hypothesis become meaningful. Actually there is really good evidence available which shows that ring glands from larvae as young as the first instar are able to promote growth in certain organ discs. More precise information concerning this point will be given in a later communication.

Summary

1. The normal development of the salivary gland of *Drosophila virilis* is described. Eleven successive stages of development have been distinguished.

2. Larval salivary glands of different ages were transplanted into the abdomens of older larvae and thus exposed prematurely to the metamorphosis factor. It was found that the metamorphosis of the transplanted glands is not autonomous but depends upon some factor in the host.

3. Glands as young as stage 3 are unable to react to the metamorphosis factor and persist as larval tissue in the adult fly. However, glands transplanted at stage 5 metamorphose synchronously with the host and hence undergo a premature metamorphosis. In these cases the transplanted larval glands are completely histolysed, and the simultaneously transplanted anlagen of the imaginal salivary gland differentiate into adult salivary glands.

4. Salivary glands of older larval donors transplanted into younger hosts metamorphose before the host glands show any signs of metamorphosis.

5. Larval salivary glands of various ages were transplanted into the body cavity of adult male flies. The thus transplanted glands ceased to grow and remained unchanged even when left for a considerable length of time in their adult environment. If, however, ring glands of old larvae are transplanted together with salivary glands into the adult host, the growth of the salivary glands is restored, leading finally to metamorphosis. These facts have been demonstrated very clearly by using salivary glands of a single donor and transplanting one partner into one host without ring glands and the other partner into a second host together with ring glands.

6. The number of ring glands implanted is of no great importance for the development of the salivary glands, since two ring glands have about the same effect as four ring glands. However, one ring gland is presumably somewhat less effective than four ring glands.

7. The rate of metamorphosis of the salivary gland in adult hosts is decidedly slower than in normal development.

8. Younger salivary glands metamorphose later than older salivary glands under the influence of the same number of ring glands.

9. Although the young glands metamorphose later, their metamorphosis is premature as far as their state of development is concerned.

10. A comparison of the time of metamorphosis of salivary glands in adult and larval hosts shows that metamorphosis proceeds much more rapidly in larval hosts in spite of the fact that in the adult host the salivary glands may be under the influence of as many as four ring glands.

11. The ring gland factor is presumably hormonal in nature, and is not species-specific.

12. The role of hormone concentration and tissue competence in the determination of the various stages of growth and differentiation in the development of the salivary glands is discussed.

LITERATURE CITED

BODENSTEIN, D., 1939a. Investigations on the problem of metamorphosis. IV. Developmental relations of interspecific organ transplants in Drosophila. *Jour. Exp. Zool.*, **82**: 1–30.

BODENSTEIN, D., 1939b. Investigations on the problem of metamorphosis. V. Some factors determining the facet number in the Drosophila mutant bar. *Genetics*, **24**: 494–508.

BODENSTEIN, D., 1942. Hormone controlled processes in insect development. *Cold Spring Harbor Symp. on Quant. Biol.*, **10**: (in press).

FUJII, S., 1936. Salivary gland chromosomes of Drosophila virilis. *Cytologia*, **7**: 272–275.

HADORN, E., AND NEEL, J., 1938. Der hormonelle Einfluss der Ringdrüse (corpus allatum) auf die Puppariumbildung bei Fliegen. *Roux Arch. f. Entw. mech.*, **138**: 281–304.

MAKINO, S., 1938. A morphological study of the nuclei in various kinds of somatic cells of Drosophila virilis. *Cytologia*, **9**. 272–282.

ROSS, F. B., 1939. The postembryonic development of the salivary gland of Drosophila melanogaster. *Jour. Morph.*, **65**: 471–495.

SONNENBLICK, B., 1940. The salivary glands in the embryo of Drosophila melanogaster. *Genetics*, **25**: 137.

VOGT, M., 1940. Zur Ursache der unterschiedlichen gonadotropen Wirkung der Ringdrüse von Drosophila funebris und Drosophila melanogaster. *Roux Arch. f. Entw. mech.*, **140**: 525–546.